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# Chemical Adjuvant Cryosurgery With Antifreeze Proteins

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**Background and Objectives:** Imaging monitored cryosurgery is emerging as an important minimally invasive surgical technique for treatment of cancer. Although imaging allows excellent control over the process of freezing itself, recent studies show that at high subzero temperatures cells survive freezing. Antifreeze proteins (AFP) are chemical compounds that modify ice crystals to needle-like shapes that can destroy cells in cellular suspensions. The goal of this study was to determine whether these antifreeze proteins can also destroy cells in frozen tissue and serve as chemical adjuvants to cryosurgery.
**Methods:** Livers from six rats were excised, perfused with solutions of either phosphate-buffered saline (PBS) or PBS with 10 mg/ml AFP-I, and frozen with a special cryosurgery apparatus. Lobes were frozen with one or two freeze–thaw cycles and the cell viability was examined with a two stain fluorescent dye test and histological assessment.
**Results:** A significant percentage of hepatocytes survive freezing on the margin of a frozen cryolesion. AFP significantly increase cellular destruction in that region apparently through formation of intracellular ice.
**Conclusions:** This preliminary study demonstrates that antifreeze proteins may be effective chemical adjuvants to cryosurgery.
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KEY WORDS: cryosurgery; liver surgery; antifreeze proteins; freezing damage; chemical adjuvant cryosurgery

## INTRODUCTION

Cryosurgery is a surgical technique that uses cryogenically cooled probes to locally freeze and thereby destroy undesirable tissues. This technique has been used for the treatment of cancerous tumors since the middle of the nineteenth century [1]. Cryosurgery has significant advantages as a minimally invasive surgical technique. Intraoperative imaging of freezing with ultrasound [2,3] and magnetic resonance imaging [4] have produced a revival in the use of cryosurgery for treatment of solid tumors deep in the body, such as liver and prostate cancer tumors [5,6]. However, while imaging can provide detailed information on the extent of the frozen tissue, it does not produce any information on how much of the frozen tissue is actually destroyed. Recent studies, however, have revealed that not all of the frozen tissue is destroyed [7,8]. A study on liver cryosurgery has found that the temperature threshold to obtain complete necrosis of normal liver cells by freezing is −15°C [7]. Experiments with prostate adenocarcinoma cells, from an ND-1 cell line, frozen in vitro, have shown that a double freeze–thaw cycle is required to destroy all cells [8]. Furthermore, even with a double freeze–thaw cycle the temperature threshold for complete damage is −20°C [8]. Studies on the relation between thermal parameters during freezing and frozen tissue damage are important because they can be incorporated into the design of optimal cryosurgical protocols. Nevertheless, the evidence of cell

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survival in the frozen region, around the outer margin of a cryolesion, is of concern. It would clearly be beneficial to find a method to enhance cell destruction in the region of frozen tissue in which cells were found to survive freezing. Recent studies suggest that ''antifreeze proteins'' (AFP) are a family of proteins that may be useful for this purpose. In this study we explore the idea that ''antifreeze proteins'' could be used to enhance frozen cell destruction during cryosurgery.

''Antifreeze proteins'' are chemical compounds produced by a variety of organisms, animals and plants, as protection from cold injury [9]. In nature, they normally depress the freezing temperature of solutions by biding to ice crystals and inhibiting their growth up to certain temperatures. Typically they depress the freezing temperature by about 0.5 to 1.0°C. However, once their inhibitory effect is breached by further lowering the temperature, these proteins modify the structure of the ice crystal to which they are bound, producing needle like crystals that can be damaging to cells. Four different types of antifreeze proteins, referred to as AFGP, AFP-I, AFP-II, and AFP-III have been identified in nature [9]. They all share the ability to dramatically modify the microstructure of ice, presumably by binding to specific ice crystal planes [9,10].

Crystallographic studies have characterized the relationship between ice crystal structure and antifreeze protein concentration [11]. These studies show that the antifreeze proteins produce prism-like ice crystal structures. The size of the prisms is concentration dependent. Needle like spicular ice crystals form at concentrations of about 5 mg/ml and higher. Lower concentrations produce larger prismatic ice crystals, with dimensions inversely related to the protein concentration. Studies with red blood cells [12,13], cardiomyocytes [14], Chinese hamster fibroblasts (V79W), lymphocytes, and granulocytes [15] have found that these proteins can enhance cell destruction during freezing. The increased damage was found to be related to the formation of spicular needle-like ice crystals in the presence of the proteins and the apparent consequent formation of intracellular ice, observed as cytoplasmic darkening [14,15]. The mechanism by which the cells are destroyed in the presence of antifreeze proteins is not yet understood.

Koushafar and Rubinsky [16] have recently reported the results of a preliminary study on the potential use of antifreeze proteins as chemical adjuvants to cryosurgery. They have examined the dose effect of AFP-I on adenocarcinoma prostate cancer cells frozen in vitro [16]. It was found that antifreeze proteins enhance the destruction of in vitro frozen cells [16]. The effect of the AFP's was found to be dose dependent. A dose of 10 mg/ml AFP-I produced complete cell damage that was independent of the thermal history the cells experience during freezing. As a second step in developing AFP adjuvant cryosurgery, we have begun to examine the effect of AFP-I on the survival of cells frozen in tissue in situ. Here we report results with simulated cryosurgery of whole liver lobes perfused with solutions containing antifreeze proteins of type AFP-I.

## MATERIALS AND METHODS

### Animals

Six adult male Sprague–Dawley rats were used in this study. The rats, which weighed from 250 to 300 grams, were obtained from Simonsen Laboratories (Gilroy, CA). All experimental procedures were performed in accordance with NIH guidelines for care and use of laboratory animals and approved by the Animal Care and Use Committee at the University of California at Berkeley.

### Solutions

Natural antifreeze proteins of type I (AFP-I) from the winter flounder, *Pseudopleuronectes americanus* (A/F Protein, Waltham, MA), were used in this study. These 3.4 kDa proteins have 37 residues, are alanine rich and are $\alpha$-helical [11]. To determine the effect of these proteins on frozen cell survival we added 10 mg/ml antifreeze proteins to a phosphate-buffered saline (PBS) solution. This concentration was chosen because the earlier study of Koushafar and Rubinsky showed that it induces maximal damage to cells frozen in suspension [16].

### Surgical Technique

Animals were provided with ample food and water prior to the surgical procedure. The rats were anesthetized with Metofane (Pitman-Moore, Inc., Mundelein, IL). The peritoneal cavity was entered via a midline incision of the abdomen. After partial mobilization of the liver from adjacent tissue and ligation of the small lobes, a 16-gauge Teflon intravenous catheter was introduced into the portal vein and 3 ml of 4°C PBS solution containing 1000 units of heparin was rapidly infused using a 3-ml syringe. The syringe was removed and the portal vein catheter was perfused with cold PBS, at 4°C, for the remainder of the procedure. The inferior vena cava was ligated above the renal vein and below the hepatic vein to prevent mixing of blood flow and perfusate and transected distally to relieve blood pressure and exsanguinate the animal. The perfusion line was removed and a 3-ml solution of cold PBS with either no AFP (control) or with 10 mg/ml AFP-I was injected into the catheter. The two largest lobes were ligated and removed separately for the freezing protocol. Smaller unligated lobes were used for unfrozen controls.

### Freezing Apparatus

An experimental setup was constructed to simulate in vitro the process of freezing during cryosurgery. The freezing apparatus, shown schematically in Figure 1,

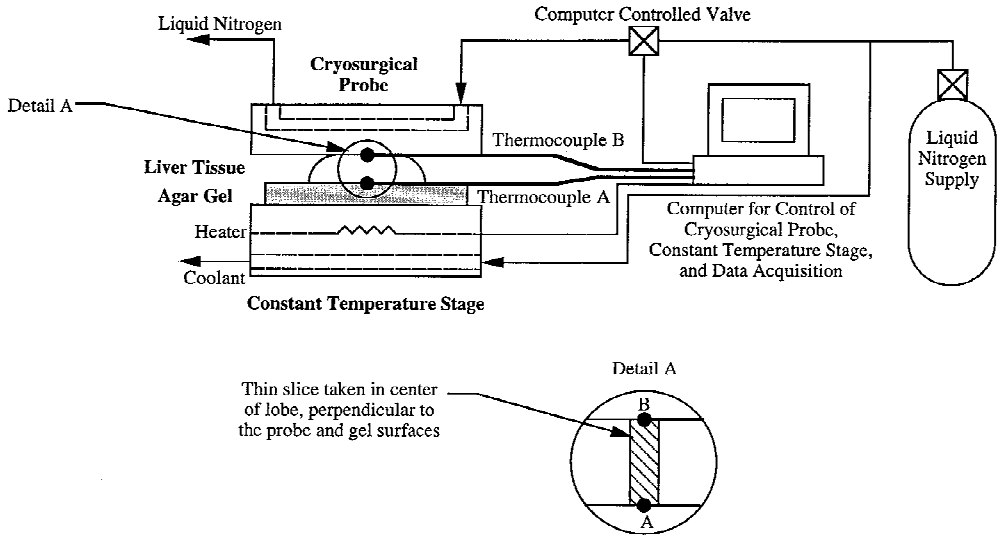


Fig. 1. Schematic presentation of the freezing apparatus showing the constant temperature stage, the cryosurgical probe, and the temperature control system. The figure also shows the liver between the cryosurgical probe and the constant temperature stage, the location of the thermocouples marked with A and B. The figure detail shows the location from which examined liver slices were taken.

consists of a constant temperature stage, a layer of agar gel, and a constant temperature cryosurgical probe.

The stage was constructed from a copper block. The copper block was cooled by liquid nitrogen perfusion and warmed by resistive heating elements. The heating elements were connected to a temperature control system, thus maintaining a constant temperature at the upper surface of the block. The details of the stage may be found in a previous report [17]. The surface of the stage could be set at a desired constant temperature throughout the experiment.

The stage surface was covered with a layer of agar gel (1 cm thick) prior to the experiment. The gel was used to approximate the thermal properties of liver tissue.

A cryosurgical probe with a circular flat quartz surface, (26 mm diameter) was used to induce freezing. Details of the probe were described previously [18]. The probe was connected to a computer control system [19]. The control system modulated the flow of cryogen through the cryosurgical probe to attain a constant temperature at the probe surface in contact with the liver.

## Experimental Procedure

Once excised, the liver lobe was immediately placed on the flat agar gel surface on the constant temperature stage. The temperature of the stage was maintained at 4°C throughout the experiment and the temperature of the agar gel was 4°C prior to the onset of freezing. A T-type thermocouple was sandwiched between the agar surface and the lobe approximately in the center of the bottom surface of the lobe (thermocouple A, Fig. 1). The thermocouple, connected to our control system, allowed us to monitor and record the temperature at the bottom surface throughout the experiment. The flat surface cryosurgical probe was set to −25°C and placed on top of the lobe. The lobe was thus sandwiched between the cryosurgical probe and the agar surface (Fig. 1). Another T-type thermocouple was fixed to the center of the cryoprobe surface to monitor the temperature of the top surface of the liver lobe (thermocouple B, Fig. 1). The surface of the cryosurgical probe was maintained at −25°C, throughout the experiment. A temperature of −25°C was chosen for these experiments because we wanted to focus our investigation on the range of temperatures in which previous studies have indicated that liver tissue survives freezing.

We performed single and double cycle freeze–thaw experiments. In the single cycle experiment, freezing was stopped when thermocouple A registered a temperature of −3°C. The lobe was then immediately separated from the probe and prepared for the viability studies. In the double-cycle experiment, the flow of cryogen through the cryo probe was halted when thermocouple A registered a temperature of −3°C. To begin the thaw cycle, the temperature of the constant temperature stage was immediately raised to 37°C. The thaw cycle was extended for 5 minutes beyond the time at which thermocouple B registered 0°C. At this point, the stage was reset to 4°C and the flow of cryogen into the cryosurgical probe was resumed. The second freezing cycle was also terminated when thermocouple A registered a temperature of −3°C. The lobe was then immediately separated from the probe to be prepared for the viability studies.

## Viability Studies

Following the removal of the frozen liver lobe from the freezing apparatus, thin liver slices were prepared for

viability analysis. A great deal of care was used to ensure that the slices were as thin as possible. Therefore, the slices were taken using a scalpel while the tissue was still frozen. The cuts were made through the liver, in a direction perpendicular to the surface of the lobe that was in contact with the cryosurgical probe. Consequently, the length of the slices corresponds to the thickness of the lobe. (Detail A in Fig. 1 illustrates how the liver is cut to obtain a slice.) The other dimensions of the slices were 2 mm wide and about 0.1 mm thick. Several thin slices were taken from the center of the lobe, as close as possible to the location of thermocouples A and B, as shown in Figure 1. Some of the slices were placed in an Eppendorff tube containing 1 ml of a cell viability assay solution and others were fixed in 10% buffered formalin phosphate (Fisher Scientific, Fair Lawn, NJ) for pathological examination. Tissue slices from a lobe that was not frozen were also prepared in a similar form. They were placed in a cell viability assay solution and in formalin to serve as controls.

The cell viability assay solution is a two-color fluorescent test that utilizes the plasma membrane integrity to differentiate between live and dead cells. The assay contains a membrane-permeant nucleic acid stain (SYTO 11®, Molecular Probes, Eugene, OR), which upon entering the cells binds to DNA molecules to produce an intense uniform green (508 nm) color. The assay also contains membrane-impermeant Ethidium homodimer (EthD-1) (Molecular Probes), which can only penetrate into cells with damaged membranes. EthD-1 produces a bright red (600 nm) fluorescence in dead cells.

To use the cell viability assay, the liver slices were incubated for 40 minutes in 1 ml of Dulbecco's phosphate-buffered salt solution containing 0.5 mM SYTO 11, 16 mM EthD-1, and less than 0.1% dimethyl sulfate. Following incubation, the slices were carefully removed from the assay solution and placed on a glass slide. The orientation of the tissue slice was maintained throughout the above procedure to help us determine the thermal history at each end of the tissue. A glass cover slip was placed on top of the tissue and gently pressed.

Images were taken with a 40× objective lens using a BH-2 Olympus microscope with fluorescence imaging capabilities, a PM-10ADS Olympus automatic photomicrographic system, and Kodak Ektachrome 400× professional color reversal film. A measurement scheme was devised to remove human bias in viability analysis. Six positions along the length (x-coordinate) of the slice were chosen for examination before the viability test was performed. The same relative x-coordinate locations were used for each of the slices and experiments. In each viability measurement the microscope ocular was brought to one of the six x-coordinate locations and, without looking through the microscope, the ocular was moved to a random y-coordinate location along with the width of the slice. The tissue was imaged at three different randomly chosen positions along the width (y-coordinate) of the slice for each of the six positions along the x-axis. Each position was imaged twice using two different fluorescent cubic units with different dichroic mirror, exciter, and barrier filters to selectively exclude either the green or red fluorescence.

The prepared slides were then used to quantify cell viability. Because SYTO 11 is a membrane-permeant nucleic acid stain, both dead and live cells fluoresce green. However, only cells with damaged membranes fluoresce red. This characteristic was used to quantify live and dead cells.

The above procedure was carried out for single-cycle and double-cycle frozen samples. For each experiment, an unfrozen control slice was also assayed using an identical procedure.

From all of our frozen and unfrozen tissues, a sample was fixed with formalin and sent to a pathology laboratory to be embedded in paraffin, sectioned to 5 μm thickness, and stained with hematoxylin for histological analysis.

## RESULTS

### Fluorescent Dye Results

Figures 2 and 3 show the percentage of dead cells, normalized and homogenized relative to baseline viability values obtained from unfrozen controls, after one and two freezing cycles, respectively. The results were obtained with the fluorescent dye assay and give the percentage of dead cells after freezing with and without antifreeze proteins at the six x-axis locations sampled along the tissue slice. Each data bar was obtained from the analysis of experiments with three different animals and represents the average of nine measurements. For each of the three animals, three measurements were taken along the y-axis of any x-axis tissue slide sampling location, as described in the previous section. The error bars give the value of one standard error or the mean. The abscissa gives distance from the lobe surface touching the cryosurgical probe to the lobe surface touching the agar gel. The distance is given as a percentage of the total length of the tissue sample, i.e., 0% corresponds to the lobe surface that was in touch with the cryosurgical probe and 100% corresponds to the lobe surface that was opposite the cryosurgical probe and in touch with the agar gel. It is well established in the heat transfer literature [20] that in the one-dimensional process of freezing used in this experiment the temperature in the frozen sample is linear between the temperature of the two thermocouples A and B. Therefore, the final freezing temperature that the tissue samples have experienced can be estimated by linear interpolation from the temperatures on thermocouples A and B. These temperatures are also given on the abscissa, in parentheses.

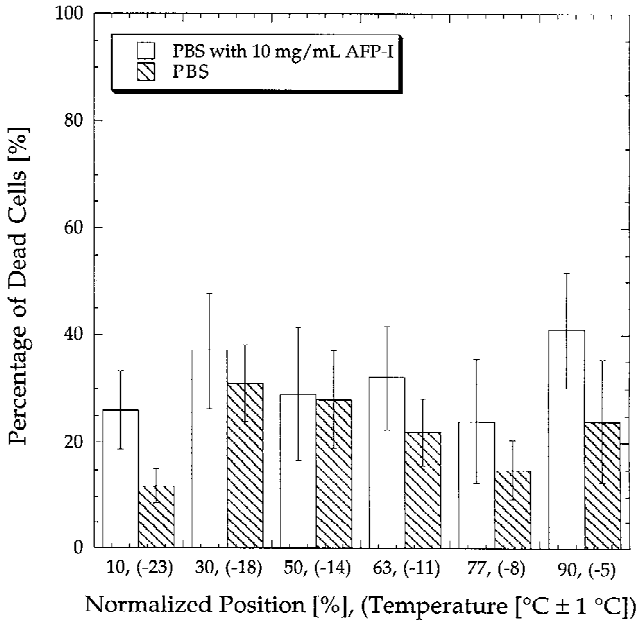


Fig. 2. Single freeze–thaw cycle. Column bars showing the average percent of cells that are necrotic after one freeze–thaw cycle with and without antifreeze proteins (AFP-I). The value of one standard deviation of the mean is also shown on the bars. Each column bar represents the results of nine measurements from three animals. The abscissa gives the location where the measurements were made. The values represent the distance from the cryosurgical probe surface, normalized with respect to the length of the liver slice. In parentheses, the abscissa gives the estimated temperature at that location. (PBS: phosphate-buffered saline).

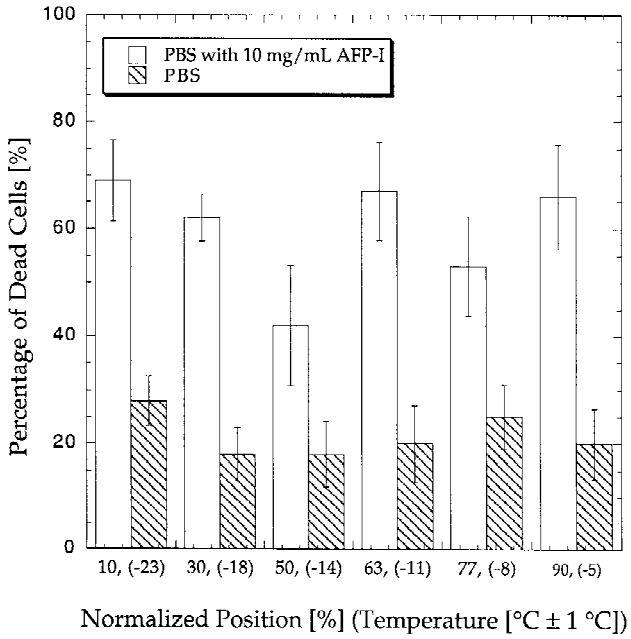


Fig. 3. Double freeze–thaw cycle. Column bars showing the average percent of cells that are necrotic after two freeze–thaw cycles with and without antifreeze proteins (AFP-I). The value of one standard deviation of the mean is also shown on the bars. Each column bar represents the results of nine measurements from three animals. The abscissa gives the location where the measurements were made. The values represent the distance from the cryosurgical probe surface, normalized with respect to the length of the slice. In parentheses, the abscissa gives the estimated temperature at that location. (PBS: phosphate-buffered saline).

We found that a surprisingly low percentage of cells, averaging between 17 and 37%, were destroyed by freezing after a single freeze–thaw cycle without AFP (Fig. 2). Cell destruction appeared to be independent of location and temperature, in the examined temperature range.

After one freeze–thaw cycle with AFPs the average percentage of cells destroyed by freezing increased to between 25 and 41% (Fig. 2). The effect of the AFP was relatively modest. Two paired *t*-tests were performed on the results of the single freeze-thaw cycle and showed that with the null hypothesis the difference between cell destruction with and without antifreeze proteins could be established with greater than 97% confidence.

In the examined temperature range, the percentage of liver cells destroyed by a double freeze–thaw cycle without AFP (Fig. 3) was statistically similar to that of liver cells destroyed by a single freeze thaw cycle without AFP. However, the average percentage of destroyed cells increased to between 42 and 70% when AFPs are used. This is more than double the percentage of cells destroyed under the same freezing conditions without AFP. The AFPs obviously have a strong effect on increasing the destruction of cells frozen with a double freeze–thaw cycle. Two paired *t*-tests were performed on the results of the double freeze–thaw cycle and show that with the null hypothesis the difference between cell destruction with and without antifreeze proteins can be established with greater than 99.999% confidence.

### Histology Results

Microscopic examination of stained tissue has produced qualitative observations on the damage caused by freezing with antifreeze proteins. These observations, illustrated by Figure 4a to e, are focused on the microscopic appearance of hepatocyte nuclei. Figure 4a shows hepatocytes from fresh livers. The round shape of the healthy hepatocyte nuclei is evident. Figure 4b and c illustrates, respectively, the appearance of liver tissue after one freeze–thaw cycle with and without antifreeze proteins. It is seen that after one freeze–thaw cycle many of the nuclei appear intact and comparable in shape and size to the nuclei in the unfrozen controls. Some of the nuclei, however, appear distorted. Figure 4d and e, illustrates the appearance of the nuclei after a double freeze–thaw cycle with and without AFP, respectively. Probably the most striking feature is the near complete destruction of hepatocyte nuclei in the presence of AFP, in Figure 4e.

## DISCUSSION

Freezing inflicts biological damage at the cellular level [21,22] and at the tissue structural level [23]. It is well

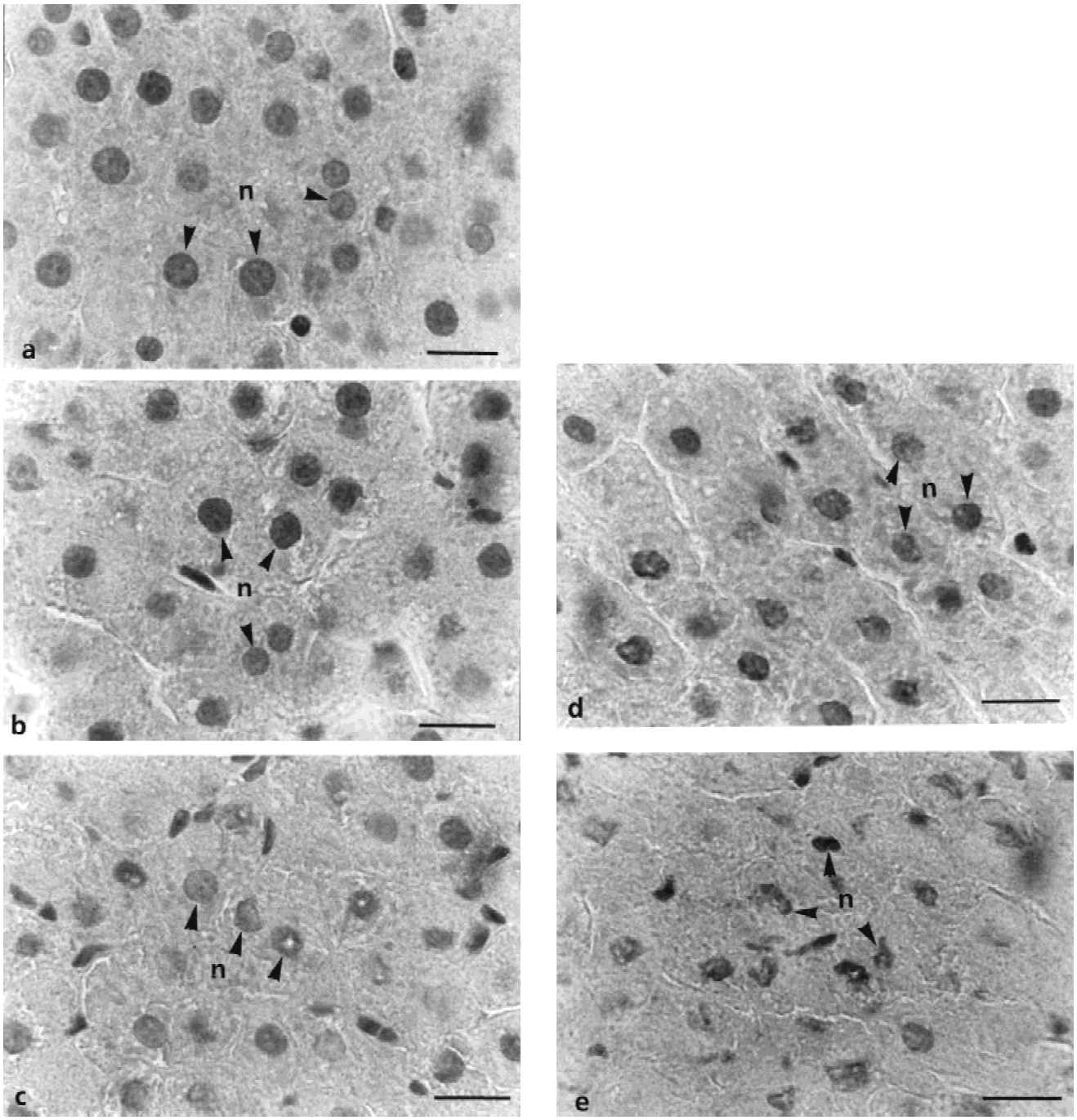


Fig. 4. Typical micrographs of liver tissue after different treatments, showing the nuclei (n): **(a)** fresh liver, **(b)** one freeze–thaw cycle without antifreeze proteins (AFP), **(c)** one freeze–thaw cycle with AFP, **(d)** two freeze–thaw cycles without AFP, **(e)** two freeze–thaw cycles with AFP. Scale bar: 20 μm. 100× magnification.

established in the cryobiology literature that at the cellular level, damage is related primarily to the cooling rates that cells experience during freezing and to the temperature to which the cells are frozen [21,22]. Mazur has explained the mechanism of cell damage by freezing through a ''two-mode'' theory [22], which suggests that during freezing with low cooling rates, ice forms first extracellularly, while the intracellular solution does not freeze and remains supercooled. To equilibrate the difference in chemical potential between the extracellular space that is in thermodynamic equilibrium and the intracellular space that is supercooled, water will leave the cell, through the water-permeable cell membrane and freeze in the extracellular space. Cell dehydration results in an increase in intracellular concentration and in consequent chemical damage to intracellular components.

Chemical damage is obviously a direct function of temperature and of the time the cells are kept at that temperature. Therefore, increasing cooling rates to lower temperatures will reduce the chemical damage caused by freezing. However, high cooling rates can cause intracellular ice to form in the supercooled cells. Mazur proposed that intracellular ice formation is also damaging. Because both chemical damage and intracellular ice formation are temperature and time related, cell damage inflicted by freezing will depend not only on the cooling rates but also on the temperature to which the cells were frozen and the time they are kept at that temperature. Therefore, the relation between cell damage by freezing and the thermal history cells experience during freezing is a complex function of cooling rates, the temperature to which the cells are frozen and the time they are kept at that temperature.

In addition to cellular damage, freezing also destroys the tissue structural integrity. The damage is caused by freezing damage to connective tissue and by cell dehydration induced accumulation of water in the extracellular interstitial and vascular spaces. The blood vessels are one of the primary sites affected by this loss of structural integrity, which results in the arrest of blood flow to the formerly frozen region and cell death by ischemia [23].

During cryosurgery, the frozen cells experience different thermal histories as a function of the temperature of the cryosurgical probe and their distance from the probe. As indicated in the introduction, this causes some cells to survive cryosurgery primarily in the region of high subfreezing temperatures. It has been found that in the whole pig liver, the tissue survives freezing at temperatures higher than −15°C [7]. In experiments with adenocarcinoma prostate cancer cells, we have found that they survive freezing to temperatures higher than −20°C for high cooling rates that produce intracellular ice, and to −35°C for lower cooling rates that produce chemical damage due to dehydration. Cells survive these conditions after single- and double-cycle freezing.

Our earlier study with ND-1 adenocarcinoma prostate cancer cells shows that antifreeze proteins can significantly increase cell destruction by freezing [16]. The mechanism by which the antifreeze proteins induce cell damage is not completely understood. It appears, however, to be related to the interaction between the needle-like spicular ice crystals and cells, and to the formation of intracellular ice under conditions in which it would otherwise not form. This is supported by the observation that with the ND-1 cells only concentrations of antifreeze proteins that cause needle-like ice crystal structures induce cell damage [16]. Further support comes from experiments in which the process of freezing in the ND-1 cells was observed with light microscopy during freezing with low cooling rates that produce chemical damage [16]. It was observed that at high subzero temperatures near the outer margin of the freezing interface the ND-1 cells were engulfed by ice but remained transparent and retained their normal shape. This indicates that there was no intracellular ice. In contrast, for the same cooling rate but in the presence of antifreeze proteins the cells became dark and froze intracellularly, close to the outer edge of the freezing interface. These results are consistent with other studies with red blood cells [12,13], cardiomyocytes [14], Chinese hamster fibroblasts (V79W), lymphocytes, and granulocytes [15]. Those studies also show enhanced cell destruction by freezing with antifreeze proteins. The damage was related to the formation of intracellular ice, observed as cytoplasm darkening, and to the presence of needle-like spicular ice crystals.

Most of the earlier studies with antifreeze proteins were done with cells frozen in suspension. However, it is not obvious from studies with individual cells that antifreeze proteins can also induce cell destruction in whole tissue. The goal of this study was, therefore, to determine whether antifreeze proteins can also induce enhanced damage of frozen cells in tissue perfused with a solution of antifreeze proteins. Figures 2 and 3 illustrate some rather surprising results. We found that after one freeze–thaw cycle, a relatively large percentage of hepatocytes survive freezing to temperatures as low as −23°C (Fig. 2). Furthermore, it appears that the extent of cell damage is relatively uniform throughout this range of temperatures. The surprisingly low percentage of cells determined to be dead may be related to the limitations of our viability test. The fluorescence test identifies only the cells that are necrotic immediately after the cryosurgical procedure. However, immediately after cryosurgery some cells can appear to be intact but may have suffered less obvious damage and, in fact, be in a state of apoptosis. Furthermore, even cells that survive freezing may later succumb to anoxia caused by cryosurgery induced loss of tissue structural integrity and damage to the vascular supply [23]. Our test does not evaluate long-term cell damage, either that due to apoptosis or due to loss of tissue structural integrity. This may explain why Rivoire et al. [7] have found that the temperature threshold for complete normal liver necrosis was −15°C, while we find a large percentage of dead cells at temperatures as low as −23°C. Figure 2 also shows that after one freeze–thaw cycle antifreeze proteins have a certain damaging effect on cells in whole frozen tissue, but this effect is small.

Figure 3 shows that a second freeze–thaw cycle does not alter significantly the percentage of necrotic hepatocytes in livers frozen without antifreeze proteins. However, the presence of antifreeze proteins has a profound effect on the destruction of hepatocytes in the frozen liver. From the data shown in Figure 3 it is possible to conclude with a confidence level of 99.999% that antifreeze protein do increase cell destruction in frozen tissue, throughout the examined temperature range.

Figure 4 suggests a possible explanation for the mechanism by which antifreeze proteins can cause cellular destruction in frozen tissue. Figure 4e, which shows the complete destruction of the hepatocytes' nuclei frozen after a double freeze–thaw cycle with antifreeze proteins, is particularly revealing. It is obvious by comparing Figure 4e with all the other parts of Figure 4 that the antifreeze proteins have affected the nuclei. Because nuclei are inside the cells, obviously the mechanism of damage relates to an intracellular event. The most likely explanation is that the antifreeze proteins produced intracellular freezing of cells in tissue, in a similar way to the intracellular freezing that was observed when cells where frozen in suspension with antifreeze proteins. The fact that antifreeze proteins have a dramatic effect on cell death during the second freeze–thaw cycle and not during the first cycle may be explained by their lack of access to the interstitial space during the first freezing cycle. Prior to the first cycle, perhaps because of our limited perfusion, the antifreeze proteins were probably restricted primarily to the vascular space. After the first freeze–thaw cycle the interstitial space has become accessible because of damage to the vasculature and, consequently, the antifreeze proteins gained access to the interstitial space. In the interstitial space the antifreeze proteins modified the structure of the ice crystals near the cells and induced the lethal formation of intracellular ice at the high subzero temperatures of this experiment.

## CONCLUSIONS

A study with liver tissue frozen in vitro with thermal conditions that emulate the process of freezing on the margin of a cryolesion demonstrates that antifreeze proteins can significantly increase cell damage during cryosurgery. The mechanism of damage appears to be related to antifreeze proteins induced formation of intracellular ice. The effectiveness of the antifreeze proteins is significantly increased during the second cycle of a two freeze–thaw cycle cryosurgical procedure. This may be explained by the greater access of the antifreeze proteins to cells during this second cycle. This study provides strong evidence that antifreeze proteins could serve as effective chemical adjuvants to cryosurgery.

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